Peer Review File

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Reviewer A

This comprehensive review will be a valuable resource for the biomedical community interested in understanding the genetic etiologies for congenital heart disease.

Minor comments are related to readability/clarity. Some examples are below. Edits are provided as suggestions.

1) 107 CNVs are large deletions or
108 duplications of DNA and pathogenic CNVs that are associated with syndromic CHD[.]
109 [These] include 22q11.2 deletion syndrome (DiGeorge syndrome) (35-41)..."
Reply 1): We have modified our text as advised (see Page 7, line 106-107).
Changes in the text: CNVs are large deletions or duplications of DNA and pathogenic CNVs that are associated with syndromic CHD. These include 22q11.2 deletion syndrome (DiGeorge syndrome) (35-41)...

2)152 Heterozygous mutations in GATA4 [italicize GATA4] were first discovered in
153 familial cardiac septal defects (70). GATA4 [italicize GATA4] mutations have been associated...
Reply 2): We have italicized GATA4 as advised (see Page 9, line 144-145).

3) 165 Interestingly, GATA6 mutations were also found to be an important
166 cause of pancreatic abnormalities (hypoplasia and agenesis) and associated Type 1 diabetes
167 mellitus (84,86). [Consider adding a mention of a recent article by Sharma et al., "GATA6 mutations in hiPSCs inform mechanisms for maldevelopment of the heart, pancreas, and diaphragm", eLife].
Reply 3): We appreciate the reviewer's important suggestion. We have modified our text and cited this article as advised (see Page 10, line 158-162).
Changes in the text: GATA6 was recently shown to function as a pioneer factor in cardiac development, regulating transcriptional activation of critical genes associated with the development of the heart as well as endodermal lineages, pancreas and diaphragm (167). These findings illuminated the molecular mechanisms for diverse developmental defects such as cardiac outflow tract defects, pancreas and diaphragm dysgenesis in patients with distinct GATA6 variants.

4) 210 Moreover,
211 genetic variants in an oligogenic manner, epigenetic changes, gene-environment
212 interactions also contribute to the development of CHD (122-124). [This sentence is not clear to me, as written.]

Reply 4): We appreciate the reviewer's comments. We have modified the text as advised to increase clarity (see Page 12, line 197 – Page 13, line 204).
Changes in the text: Although a significant proportion of CHD cases are likely to have some environmental etiologic contribution, it has been difficult to quantify the specific role these environmental contributors play in disease development. The underlying mechanisms by which environmental factors disrupt molecular pathways during cardiac development to cause CHD remain unknown. Moreover, CHD has been shown to be caused by gene-environment interactions in mice, where haploinsufficiency of Notch1 in developing embryos together with hyperglycemic or hypoxic exposure resulted in increased incidence of CHD (182-184).

5) 259 Recessive genotypes in MYH6 [italicize MYH6] were identified ~11% of patients...
Reply 5): We have italicized MYH6 as advised (see Page 15, line 248).

6) 272 RBFOX2 has a variety of biological activity
273 contributing [to] neuronal maturation and axon assembly...
Reply 6): We have modified our text as advised (see Page 16, line 259-260).
Changes in the text: RBFOX2 has a variety of biological activity contributing to neuronal maturation and axon assembly...

7) 288 Although NGS technologies in CHD cohorts have contributed to identifying causative
289 variants associated with CHD... [As the authors suggest in subsequent sentences, it is not clear that these variants are causative. Please remove "causative," and consider: "contributed to identifying
289 variants associated with CHD [risk]...
Reply 7): We have modified our text as advised (see Page 17, line 273-274).
Changes in the text: Although NGS technologies in CHD cohorts have contributed to identifying variants associated with CHD risk...

8) 292Third,
293 with the increased availability and use of WGS [,] the role of noncoding variation in the
294 genetic architecture of CHD is not clear.
Reply 8): We have added "," in the text as advised (see Page 17, line 277).

9) 306 In
307 these studies, variants were filtered using bioinformatics pipelines and prioritized
308 according to in silico prediction programs, predicted mode of inheritance, minor allele
309 frequencies, and presence [delete "of", replace with "in"] databases such as dbSNP...
Reply 9): We have replaced with "in" from "of" in the text as advised (see Page 18, line 291).

10) Furthermore, a 2-tiered
316 exome sequencing analysis approach has been reported that variant filtration utilizing a list
317 of 90 CHD gene identified pathogenic variants in 10% of CHD families and a
318 comprehensive family-based variant screening procedure identified candidate variants in
319 33% of families (144). [The meaning of this sentence is not clear. Please revise.]
Reply 10): We appreciate the reviewer's comments. We have modified our text as advised to increase clarity (see Page 18, line 302-307).
Changes in the text: Recently, Szot et al (205) reported the utility of their dual approaches for analyzing exome sequencing data to identify likely pathogenic variants. They achieved overall high diagnostic rate in families with sporadic and familial CHD by interrogating high confidence CHD-causing genes as well as an unbiased screen in which the exome sequencing data were analyzed comprehensively for additional variants not identified through the CHD gene list.

11) 328 Additionally, scRNA-seq has been
329 used to elucidate the mechanisms regulating the emergence and segregation of the early
330 cardiac lineage during heart development (148). [Consider adding this reference: Zhang et al., 2021, Unveiling Complexity and Multipotentiality of Early Heart Fields," Circulation Research.]
Reply 11): We appreciate the reviewer's suggestion. We have added this reference as advised (see Page 19, line 317).

12) 369 [a] NKX2-5 variant [delete "'s"] as a genetic modifier (124).
Reply 12): We have added "a" and deleted "s" in the text as advised (see Page 21, line 350-351).
Changes in the text: a NKX2-5 variant as a genetic modifier (186).

13) 390 Future studies of noncoding
391 variants identified in CHD large cohorts are needed to investigate complex genetic
392 architectures of CHD and to establish transcriptional effects of noncoding variants together
393 with other factors such as coding variants, epigenomics and oligogenic mechanisms. [Sentence is not clear. Please revise.]
Reply 13): We appreciate the reviewer's comments. We have modified our text as advised (see Page 22, line 369-371).
Changes in the text: Future studies are needed to establish the transcriptional and post-transcriptional regulatory effects of noncoding variants on cardiac development.

14) 400 benefits of genetic testing for patients with CHD include establishing [a] genetic diagnosis,

Reply 14): We have added “a” in text as advised (see Page 22, line 377-378).
Changes in the text: benefits of genetic testing for patients with CHD include establishing a genetic diagnosis,

15) 405 testing for specific syndromes are currently utilized as [delete "a"] standard genetic testing for
Reply 15): We have deleted “a” in the text as advised (see Page 23, line 382-383).
Changes in the text: testing for specific syndromes are currently utilized as standard genetic testing for

16) Initial successful examples of
422 linking genetic information to clinical outcomes were investigating the effect of common
423 single nucleotide polymorphisms instead of rare pathogenic variants. [Please clarify and revise this sentence.]
Reply 16): We appreciate the reviewer’s comments. We have modified our text as advised (see Page 23, line 396 – Page 24, line 398).
Changes in the text: Initial successful examples of linking common genetic variants for clinical care were limited to areas of prediction of disease risk, disease classification, and drug response (226).

17) Pathogenic variants in cilia genes may [delete "be poor", replace with "predict"] postoperative and respiratory
Reply 17): We have modified our text as advised (see Page 24, line 414 to Page 25, line 415).
Changes in the text: Pathogenic variants in cilia genes may predict postoperative and respiratory

18) Recent large-scale
450 study of exome sequencing in 2517 patients with CHD demonstrated consistent findings
451 (170). Clinically significant DNVs were identified in 11.7% of CHD patients and patients
452 with DNVs had more extra-cardiac anomalies. DNVs were associated with worse
453 transplant-free survival and worse postoperative respiratory outcomes in patients
454 undergoing cardiac surgery as well as patients without extra-cardiac anomalies. [There are important concepts here, but it is difficult to understand for the reader. Please revise.]
Reply 18): We appreciate the reviewer’s important comments. We have modified our text as advised (see Page 25, line 421-432).
Changes in the text: A recent study of exome sequencing in a large cohort of 2517 patients with CHD demonstrated that 11.7% of patients carried clinically significant DNVs and patients with DNVs were more likely to have extra-cardiac anomalies (233). This study also found that DNVs were associated with lower transplant-free survival

and worse postoperative respiratory outcomes such as longer times on the ventilator in patients who underwent open-heart surgery. Interestingly, the magnitude of the association between DNVs and clinical outcomes was shown to be different for patients with versus without extra-cardiac anomalies. In patients with extra-cardiac anomalies, the association of DNVs with worse outcomes was modest without statistical significance. In contrast, DNVs were strongly associated with adverse outcomes among patients without extra-cardiac anomalies. These important findings suggest a benefit for genetic testing even in patients without extra-cardiac anomalies who are not suspected to have genetic abnormalities in routine clinical practice.

19) 458 Although only a few [delete "of"] HLHS candidate genes
Reply 19): We have deleted “of” in the text as advised (see Page 26, line 433).
Changes in the text: Although only a few HLHS candidate genes

20) 460 harboring pathogenic CNVs [replace "was" with "were"
461 reported to be associated with significantly
Reply 20): We have modified our text as advised (see Page 26, line 435-436).
Changes in the text: harboring pathogenic CNVs were reported to be associated with significantly

21) 463 Patients harboring MYH6 [italicize MYH6]
464 variants had abnormal myocardial physiology and
Reply 21): We have italicized MYH6 in the text as advised (see Page 26, line 438).

22) 474 It is becoming progressively clear that continued understanding of the genetic contributors
475 to CHD is providing novel insights into not only the etiology of CHD but also increasing
476 our knowledge on how genetic variation impacts the clinical outcomes of patients with
477 CHD. [Please revise, as this is a little difficult to follow.]
Reply 22): We appreciate the reviewer’s comments. We have modified our text as advised (see Page 26, line 448-449).
Changes in the text: The increased understanding of genetics of CHD is providing new insights into the etiology of CHD as well as the impact of genetic variants on clinical outcomes in patients with CHD.

23) 477 [delete "With the"] More accurate detection and interpretation of pathologic genetic variants in
478 CHD patients will enable...
Reply 23): We have deleted “With the” in our text as advised (see Page 26, line 449-450).
Changes in the text: More accurate detection and interpretation of pathologic genetic variants in CHD patients will enable…

24) 483 Current progress in
484 sequencing technologies and functional genomic models of CHD as well as the integration
485 of precision genome editing, single-cell genomics, machine learning, cardiac
486 bioengineering and cardiac organoid models, will open doors for new regenerative and
487 preventive therapeutic approach to treat the core disease mechanisms in CHD patients in
488 the future. [Please revise this final sentence. Consider breaking up the concepts to convey message with more clarity.]
Reply 24): We have modified our text as advised (see Page 27, line 459-463).
Changes in the text: Current advances in sequencing technologies and functional genomic models of CHD will allow for the integration of genome editing, cardiac bioengineering and cardiac organoid models. The maturation of these technologies will open the door for new regenerative and preventive therapeutic approaches to treat the core disease mechanisms in CHD patients in the future.

Reviewer B
The review covers the genetics of congenital heart disease (CHD). The manuscript describes the topic thoroughly in a very structural way that is easily to follow as a reader. Especially, I am very fond of the tables that give a good summary of genetic variants associated with CHD. I only have minor suggestions.

Minor comments:
Comment 1: p.2. l.46-59: Briefly mention the differences/similarities between genetically caused CHD (especially non-syndromic CHD) and inherited cardiac diseases.
Reply 1: We have modified our text as advised (see Page 4, line 51-55).
Changes in the text include the revised sentence: While there have been significant advances in the elucidation of the genetic etiologies for other forms of inherited cardiac disease such as cardiomyopathy and arrhythmias, it has only been with the increased understanding of the molecular pathways regulating cardiovascular development over the past couple of decades that the genetic basis of CHD has become more defined.

Comment 2: p.3. l.64: oligogenic origins -> oligogenic or polygenic origins.
Reply 2: We have modified our text as advised (see Page 5, line 60-61).
Changes in the text: oligogenic or polygenic origins

Comment 3: p.3. l.85-91: It would be lovely to have the search termed used for the pubmed literature search.
Reply 3: We have modified our text as advised (see Page 6, line 84-89).
Changes in the text: Search terms included congenital heart defects in combination with genetics, etiology, pathogenesis, mutations/genetic variation, environmental factors,

next-generation sequencing, exome sequencing, whole genome sequencing, variant prioritization, single-cell RNA sequencing, functional genomics, genetic animal models, human induced pluripotent stem cells (iPSCs), noncoding variants, or genetic testing and a combination of congenital heart disease, genetics and clinical outcomes.

Comment 4: p.10. l.296: I would like to have more information on the guidelines used for variant evaluation in the different studies as this is of great importance.
Reply 4: We appreciate the reviewer's important comments. We have modified our text and cited this article as advised (see Page 18, line 295-302).
Changes in the text: Variants were then classified based on the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) guidelines (204). Since the release of these guidelines, several online tools and repositories have been developed for classification and interpretation of genetic variants, including the National Institutes of Health-funded Clinical Genome Resource (ClinGen) (https://clinicalgenome.org/working-groups/sequence-variant-interpretation/), Varsome (https://varsome.com/), Franklin (https://franklin.genoox.com/clinical-db/home) and Clinvar (https://www.ncbi.nlm.nih.gov/clinvar/).

Comment 5: p.16. l.473: The future direction section could benefit from a sub-section describing or referring to guidelines for variant evaluation, e.g. ACMG/AMP guidelines.
Reply 5: We have modified our text as advised (see Page 27, line 452-454).
Changes in the text: Further refinement of the clinical variant interpretation framework such as ACMG/AMP guidelines will construct a more accurate, consistent and transparent approach to variant classification.